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The novel interaction between microspherule protein Msp58 and ubiquitin E3 ligase EDD regulates cell cycle progression

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ABSTRACT

Microspherule protein Msp58 (or MCRS1) plays a role in numerous cellular processes including transcriptional regulation and cell proliferation. It is not well understood either how Msp58 mediates its myriad functions or how it is itself regulated. Here, by immunoprecipitation, we identify EDD (E3 identified by differential display) as a novel Msp58-interacting protein. EDD, also called UBR5, is a HECT-domain (homologous to E6-AP carboxy-terminus) containing ubiquitin ligase that plays a role in cell proliferation, differentiation and DNA damage response. Both in vitro and in vivo binding assays show that Msp58 directly interacts with EDD. Microscopy studies reveal that these two proteins co-localize in the nucleus. We have also found that depletion of EDD leads to an increase of Msp58 protein level and extends the half-life of Msp58, demonstrating that EDD negatively regulates Msp58's protein stability. Furthermore, we show that Msp58 is upregulated in multiple different cell lines upon the treatment with proteasome inhibitor MG132 and exogenously expressed Msp58 is ubiquitinated, suggesting that Msp58 is degraded by the ubiquitin–proteasome pathway. Finally, knockdown of either Msp58 or EDD in human lung fibroblast WI-38 cells affects the levels of cyclins B, D and E, as well as cell cycle progression. Together, these results suggest a role for the Msp58/EDD interaction in controlling cell cycle progression. Given that both Msp58 and EDD are often aberrantly expressed in various human cancers, our findings open a new direction to elucidate Msp58 and EDD's roles in cell proliferation and tumorigenesis.

1. Introduction

Microspherule protein 58 kDa (Msp58, also known as MCRS1) was originally identified as a nucleolar protein, and, consistent with that localization, it has since been found to regulate transcription of rRNA genes [1,2]. Msp58 is also found within the nucleoplasm, however, and is now recognized to play a role in many other cellular processes [2–6]. For example, several studies have shown that Msp58 binds to individual transcription factors, modulating their effects on target genes [2,4,5]. Msp58/MCSR1 also binds to the minus ends of kinetochore microtubules, preventing depolymerization by microtubule depolymerase and thereby modulating the stability of mitotic spindles [7]. Msp58 may have additional roles in cell cycle regulation through its transcriptional effects on the cyclin genes [8]. Interestingly, a recent study indicates that ectopic expression of Msp58 affects the protein levels of important regulators of cell cycle, including cyclins, and has distinctive effects in cell proliferation in a cell context-dependent manner [9].

The Msp58 gene is evolutionarily conserved from flies to humans [1,3,10]. Current understanding of the functions of Msp58, however, have come mostly from studies focused on other proteins, where these proteins were used as "bait" and then Msp58 identified as a binding partner in yeast two-hybrid screens. In this study, we focused on Msp58, looking for binding partners that might reveal more about the natural cellular functions of this highly conserved protein. Upon immunoprecipitating Msp58 from HeLa S3 nuclear extracts, we found several potential interacting proteins, but by far the most abundant was the E3 ubiquitin ligase EDD (E3 identified by differential display).

EDD (also called hHYD or UBR5) is an ortholog of *Drosophila melanogaster* Hyd, an E3 ubiquitin-protein ligase encoded by tumor suppressor gene *hyperplastic discs* (*hyd*) [11,12]. EDD is a nuclear protein that belongs to the family of HECT domain-containing (homologous to E6-AP carboxy-terminus) ubiquitin ligases. Like Msp58, EDD plays a role in cell cycle regulation [13–15]. EDD also has important functions in the DNA damage response [13–18].

In the current study, we show, by immunofluorescence and confocal microscopy, that Msp58 and EDD co-localize in the nucleoplasm. Our domain analyses reveal a direct association of Msp58 with EDD via a region upstream of EDD's E3 ligase domain and demonstrate that two independent regions of Msp58 are sufficient to bind EDD. Depletion of

Abbreviations: Msp58, microspherule protein 58 kDa; EDD, E3 identified by differential display; HECT-domain, homologous to E6-AP carboxy-terminus-domain; FHA, forkhead-associated domain; NLS, nuclear localization signal; siRNA, small interfering RNA; IP, immunoprecipitation

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EDD results in an increase of Msp58 protein level and stability, suggesting that EDD is a negative regulator of Msp58 (EDD's ubiquitin ligase activity may or may not be involved). Interestingly, additional experiments suggest that the Msp58/EDD interaction serves to control aspects of cell cycle progression. Upon silencing Msp58 alone, there was a pronounced depletion of Msp58 protein and this was accompanied by an increase in both cyclin D3 and cyclin B levels, leading to a stall at G2/M. Depletion of EDD alone or in combination with Msp58 provided further evidence for the importance of these two proteins and their relative expression levels to normal cell cycle progression.

2. Materials and methods

2.1. Cell Culture

HeLa and HEK293T cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. WI-38 cells were maintained in ATCC Minimum Essential Medium Eagle supplemented as described above.

2.2. Cycloheximide (CHX) and MG132 treatment

WI-38, HeLa and HEK293T cells were treated with 20 μg/ml cycloheximide (Sigma; diluted in ethanol) in the presence or absence of 40 μM MG132 (BostonBiochem; diluted in DMSO) for 6 h before harvest. Diluent (ethanol/DMSO) was used as control.

2.3. Antibodies

Rabbit anti-Msp58 serum was raised against GST-Msp58 (Reamstown, PA) and the antibodies were purified using recombinant FLAG-Msp58. Rabbit anti-EDD serum was raised against recombinant EDD (amino acids 2475–2799) fragment (Reamstown, PA) and the antibodies were purified using GST-EDD (amino acids 2475–2799). Mouse anti-EDD serum was raised against recombinant GST-EDD (amino acids 2475–2799). Commercial antibodies used in this study were as follows: FLAG (Sigma); EDD (M-19), His_6 tag, GFP and GAPDH (Santa Cruz); S tag (Novagen); cyclins B and E (BD Biosciences); cyclins D1 and D3 (Cell Signaling); and mAb414 (Covance).

2.4. Small interfering RNAs (siRNAs) and transfection

siRNAs targeting EDD (GenBank accession number NM_015902) were designed using the Whitehead siRNA design program (http://jura.wi.mit.edu/siRNAext/). The sequences of the effective EDD siRNAs used in this study are as follows: sense strand 5′-GGCAAAUCCAG AAGUGUCAdTdT, antisense strand 5′-UGACACUUCUGGAUUUGCCdTdT. The siRNAs were synthesized and processed by Dr. Thomas Tuschl laboratory at the Rockefeller University. The siRNAs against Msp58 (GenBank accession number AF015308) were designed using the Integrated DNA Technologies (IDT) SciTools RNAi Design program and were synthesized by IDT. The sequences are as follows: sense strand 5′-GUGGCAGGUGCUAGUGGACAGCATC and antisense strand 5′-GAUG CUGUCCA CUAGCACCUGCCACUU. GFP siRNAs (Dharmacon) were used as control siRNA. For WI-38, ~1×10^5 cells/well were seeded in 6 well plates the day before transfection. The cells were transfected with 100 nM siRNAs using Lipofectamine RNAiMAX (Invitrogen) and the medium was changed 5 h later. After a 48-h incubation, cells were trypsinized and transferred to 6 cm plates. For HeLa, ~4×10^5 cells/well were seeded in 6 well plates and transfected as described above. About 6 h post-transfection, cells were trypsinized and re-seeded in 6 cm plates. For HEK293T, ~1.5×10^6 cells/well were seeded in 6 cm plates. Cells were transfected with 100 nM siRNAs using calcium phosphate method as previously described [19]. Seventy-two hours after transfection, WI-38, HeLa and HEK293T cells were harvested and analyzed accordingly.

2.5. Plasmids

Full-length human Msp58 coding sequence was amplified from HeLa cDNA (Clontech) and cloned into *pIRESneo* (Clontech) for expressing FLAG-Msp58. The Msp58 coding sequence was cloned into *pEGFP-C1* (BD Biosciences), *pGEX-4T-3* (GE Healthcare), and *pET28hx* (a gift from Dr. T. Schwartz) to express GFP-Msp58, GST-Msp58 and His_6-Msp58-FLAG, respectively. Different cDNAs coding for human EDD fragments (EDDFR12, EDDFR35, EDDFR45, EDDFR1, EDDFR4 or EDDFR5) were obtained by PCR from human testis cDNA library (Clontech) and then cloned into the Not I/Apa I sites of *pcDNA3.1$^+$-HisB* (Invitrogen). The cDNAs for EDDFR4 and EDDFR5 were also cloned into the Nde I/Xho I sites of *pETDuet-1* (Novagen) to express C-terminal S tagged EDDFR4-S and EDDFR5-S. The cDNAs for Msp58 and its derivatives were amplified by PCR from FLAG-Msp58 expression vector and cloned into the EcoR I/Hind III sites of aforementioned EDDFR4-S or EDDFR5-S expression constructs to co-express N-terminal His_6-tagged Msp58 and its derivatives. All the constructs were verified by DNA sequencing. HA-Ub and FLAG-EDD expression constructs were generously provided by Drs. P. Zhou and D. Saunders, respectively.

2.6. Cell line establishment and affinity purification of protein complexes

HeLa S3 cells grown in DMEM with 10% FBS were transfected with the FLAG-Msp58 expression construct using Effectene Transfection reagent (QIAGEN) according to the manufacturer's instruction. G418 (500 μg/ml) was added to the medium for selection [20]. Positive clones were confirmed by immunoblotting the lysate using anti-FLAG antibody (Sigma). Preparation of nuclear extracts and affinity purification were carried out by following the protocol previously described [20]. Briefly, nuclear extracts prepared from FLAG-Msp58 stably transfected HeLa S3 cells were adjusted to contain 0.2% NP-40 and incubated with anti-FLAG M2-agarose beads (Sigma) at 4 °C for 6 h. After extensive washing with BC300 buffer (20 mM HEPES, pH 7.9, 300 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol) containing 0.2% NP-40, the associated complexes were eluted from beads by incubating at 4 °C for 60 min with BC100/0.2% NP-40 containing 0.5 mg/ml FLAG peptide (Sigma).

2.7. Mass spectrometric protein identification

The affinity-purified proteins were resolved by SDS-PAGE. The protein bands that were specific to the eluate of the FLAG-Msp58 sample, compared with the HeLa S3 control, were excised from a Coomassie blue stained gel, digested with trypsin, followed by protein identification by liquid chromatography–tandem mass spectrometry (LC-MS/MS) carried out in ProtTech (Norristown, PA).

2.8. Protein expression and binding assays

Recombinant S-tagged EDD fragments and His_6-tagged Msp58 were co-expressed from *pETDuet-1* vector (Novagen) in bacteria *BL21 (DE3) CodonPlus (RPIL)* (Agilent) and purified either using cobalt beads (GE Healthcare) or S-tag agarose (Novagen). GST-Msp58 was expressed in bacteria *BL21* (*DE3*) and purified using Glutathione Sepharose 4B (GE Healthcare). The purifications were performed according to the manufacturer's manuals. The EDD fragments used in Fig. 2B were produced using the TNT T7 quick coupled transcription/translation system (Promega) in the presence of ^{35}S-Met (ICN, Irvine, CA). For each reaction of binding assay in Fig. 2B, 20 μl of beads immobilized with recombinant GST-Msp58 (or GST) were incubated with *in vitro* translated protein in 36 μl of TBT buffer (20 mM Hepes, pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 0.1% Tween 20) containing complete EDTA free protease

inhibitors (Roche) at 4 °C for 1 h. After three washes, the bound proteins were eluted with SDS-sample buffer and resolved by SDS-PAGE, followed by autoradiography.

2.9. Immunoblotting

Immunoblotting was carried out as previously described [21], except that cell lysates were prepared using ProteoJET Mammalian Cell Lysis Reagent (Fermentas) or M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), containing protease inhibitors (Calbiochem), following the manufacturer's protocol. GAPDH was used as loading control.

2.10. Immunofluorescence and confocal microscopy

GFP-Msp58 expression plasmid was transfected into HeLa cells using Lipofectamine-2000 reagent (Invitrogen). HeLa and WI-38 cells grown on coverslips were fixed, permeabilized and immunolabeled following protocols previously described [21]. FITC-conjugated goat anti-mouse and Cy5-conjugated donkey anti-rabbit IgG antibodies (Jackson ImmunoResearch) were used. Samples were analyzed using a Leica TCS SP2 confocal microscope in the Bio-Imaging Facility at Hunter College.

2.11. Immunoprecipitation

HEK293T cells were seeded in 10 cm plates (~6×10^6 cells/plate). Next day, cells were co-transfected with FLAG-EDD in combination with either GFP-Msp58 or GFP expression constructs, using calcium phosphate method as previously described [19]. Forty-eight hours post-transfection, cells were washed once and harvested with PBS (137 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The cell pellet was resuspended in 1 ml PBS and split for input (100 μl) and immunoprecipitation (IP; 900 μl). For input samples, cells were pelleted and lysed with 80 μl of M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), containing protease inhibitors, following the manufacturer's protocol. IP was carried out following Sigma's Anti-FLAG M2 Affinity Gel protocol. For IP samples, cells were pelleted and resuspended in 1 ml of 1% Triton X-100 lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors). Cell suspensions were then shaken at 1.2K rpm at 4 °C for 10 min and sonicated three times (5 s each), followed by centrifugation at maximum speed for 25 min at 4 °C. Cell lysates were incubated with 20 μl beads of anti-FLAG M2 affinity gel for 1 h at 4 °C with rotation. The beads were then washed four times with 1 ml TBS (50 mM Tris–HCl, 150 mM NaCl, pH 7.4). Immunoprecipitates were eluted in 40 μl of 3XFLAG peptide solution (150 ng/μl) and then analyzed by immunoblotting with specified antibodies.

For *in vivo* ubiquitination assay, HEK293T cells were seeded at ~4×10^6 cells per 10 cm plate the day before transfection. Cells were co-transfected with HA-Ub and GFP-Msp58 (or GFP) expression constructs and EDD or control siRNAs (Reverse Lamin siRNA, Dharmacon) using calcium phosphate method. Seventy-two hours after transfection, fresh medium containing MG132 was added and cells were incubated for additional 6 h before harvesting. Cells were harvested and the input samples were prepared as aforementioned. Immunoprecipitation was carried out as previously described with some modifications [19]. Specifically, cells were pelleted, resuspended in 300 ul of 1% SDS lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% SDS, and protease inhibitors), sonicated two times (10 s each), and boiled at 80 °C for 30 min, followed by centrifugation at the maximum speed for 25 min at 4 °C. Lysates were diluted (1:10) in NP40 lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% NP40, and protease inhibitors) and incubated with 25 μl of anti-HA affinity matrix (Sigma) for 1 h at 4 °C with rotation. The beads were then washed four times with 1 ml of binding buffer. Immunoprecipitates were eluted in 50 μl of 2× SDS-PAGE sample buffer. The inputs and eluates were analyzed by immunoblotting as described.

2.12. Msp58 protein half-life analysis

Seventy-two hours after transfection with EDD or GFP siRNAs (as described in Section 2.3), HeLa cells were either left untreated or treated with cycloheximide for the indicated time before harvesting. The samples were analyzed by immunoblotting with specified antibodies. Immunoblots were quantified using ImageJ software. Msp58 protein was compared with loading control (GAPDH). The Msp58/GAPDH ratio at $t=0$ was arbitrarily set to 1, and the ratios of remaining time points were normalized to the value of $t=0$.

2.13. Flow cytometry

Seventy-two hours after transfection with siRNAs, subconfluent WI-38 cells were harvested by trypsinization and fixed overnight in cold 70% ethanol at 4 °C. The cells were then washed and resuspended in a PBS solution containing 50 μg/ml propidium iodide (PI), 200 μg/ml RNase A, 2 mM EDTA and 0.1% Triton X-100, incubating at 37 °C for 30 min. Labeled cells were analyzed using Becton Dickinson FACScalibur system in the Flow-Cytometry Facility at Hunter College. Cell cycle population distribution was analyzed with DNA analysis software MultiCycle AV (Phoenix Flow Systems).

3. Results

3.1. EDD is a novel interacting protein of Msp58

To elucidate the molecular mechanisms by which Msp58 carries out its functions, we used immunoprecipitation (IP) to isolate the proteins that associate with Msp58 *in vivo*. We first generated a HeLa S3 cell line, designated HeLa/FLAG-Msp58, that stably expresses FLAG-tagged Msp58. Using anti-FLAG M2 agarose, FLAG-Msp58 and its interacting proteins were immunoprecipitated from the nuclear extract of HeLa/FLAG-Msp58 cells. The nuclear extract of the parental HeLa S3 cells was used as a control. The eluates of the IP were resolved by SDS-PAGE and visualized by silver staining. As shown in Fig. 1A, the eluate from HeLa/FLAG-Msp58 cells (lane 2) yielded a prominent protein band larger than 250 kDa. The band containing this protein was identified by tandem liquid chromatography mass spectrometry (LC-MS/MS) to be EDD (E3 identified by differential display). In total, 19 peptides matching EDD were identified, covering 10.5% of 2799 amino acids of EDD (Fig. 1A). EDD is a member of the HECT ubiquitin-protein ligase family and plays a critical role in cell proliferation and differentiation, as well as in the DNA damage response [11,17,18,22,23]. By immunoblotting the eluates with an EDD antibody (Santa Cruz), we confirmed that EDD was specifically copurified with FLAG-Msp58 (Fig. 1B). In addition, we carried out a reverse IP to test whether Msp58 could be pulled down by EDD. This IP was performed using lysates of HEK293T cells co-transfected with FLAG-EDD and GFP-Msp58 (or control GFP) expression constructs. The eluates were analyzed by immunoblotting using antibodies against both Msp58 (lanes 3 and 4) and GFP (lanes 7 and 8) (Fig. 1C). The results clearly indicate that GFP-Msp58, but not GFP, co-purified with FLAG-EDD. Thus, we have identified EDD as a novel Msp58-associated protein.

3.2. Msp58 interacts with EDD in vitro via a region upstream of the HECT domain

To determine which region(s) of EDD mediates its interaction with Msp58, we carried out domain analyses. As shown in Fig. 2A, EDD contains a ubiquitin-associated (UBA) domain, three nuclear localization signals (NLSs), a cysteine/histidine-rich putative zinc finger

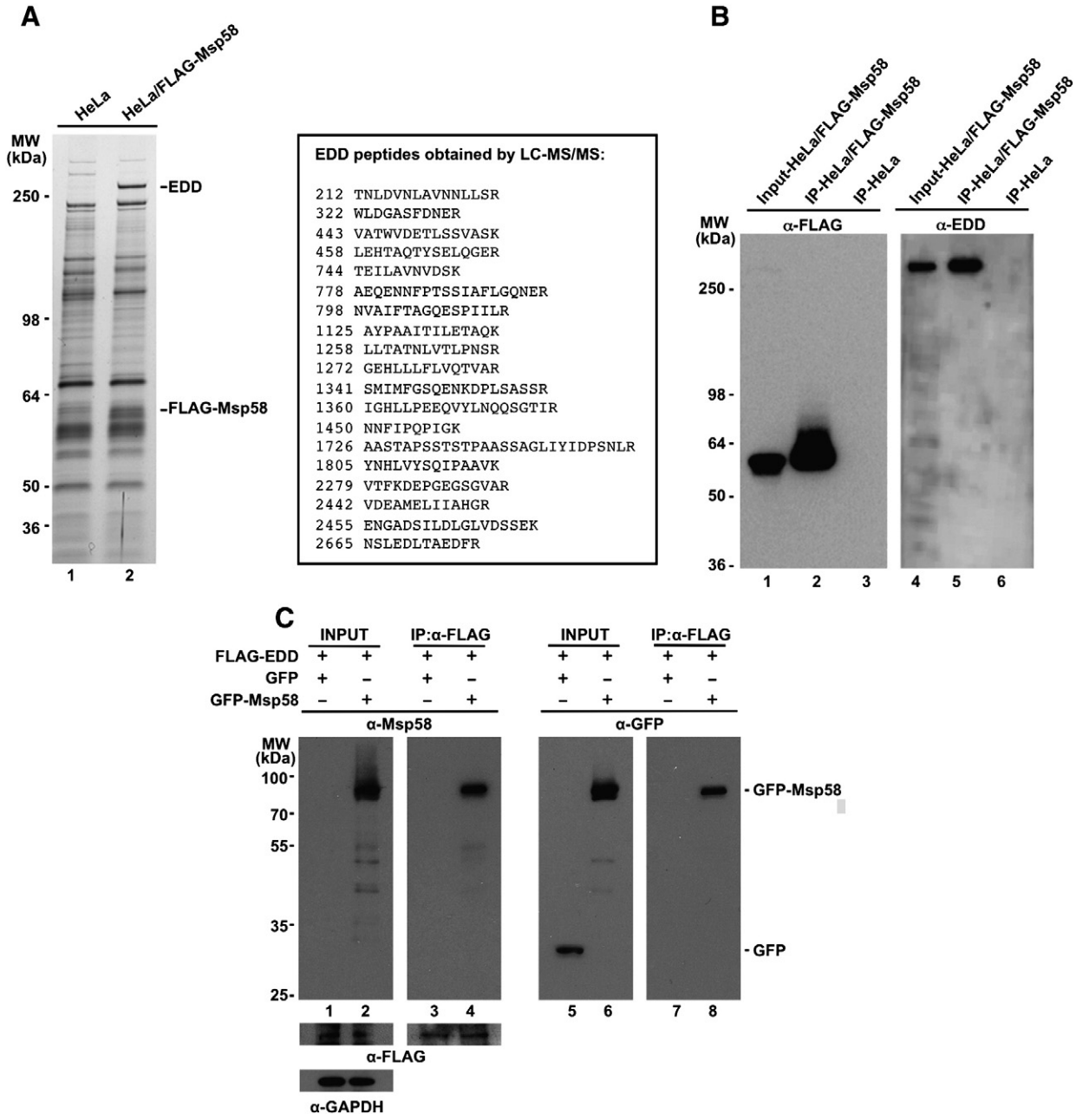

Fig. 1. Identification of a novel nuclear protein complex containing Msp58 and EDD. (A) Nuclear extract from HeLa S3 cells or HeLa S3 cells stably expressing FLAG-Msp58 was immunoprecipitated with anti-FLAG M2 agarose. The eluates were examined by silver staining. EDD was identified by mass-spectrometry (LC-MS/MS). (B) The eluates were immunoblotted with anti-FLAG and anti-EDD antibodies. Lanes 1 and 4: 5% of input, lanes 2 and 5: eluate from IP of the nuclear extract of the FLAG-Msp58 HeLa S3 stable line, and lanes 3 and 6: eluate from IP of the nuclear extract of HeLa S3 cells (control). (C) Msp58 was co-purified with FLAG-EDD. Total lysates from HEK293T cells transiently expressing FLAG-EDD and GFP-Msp58 were immunoprecipitated with anti-FLAG M2 agarose. Cells co-transfected with FLAG-EDD and GFP were used as control. Inputs (lanes 1, 2, 5 and 6) and 3X-FLAG peptide eluates (lanes 3, 4, 7, 8) were analyzed by immunoblotting with specified antibodies.

domain (zfUBR), a carboxyl region poly (A)-binding protein (PABP)-like domain (PABP-C) and a HECT domain (ubiquitin ligase domain) [17]. Fragments carrying different subsets of these domains were tested for their binding to Msp58. These included EDDFR12 (amino acids 1–1141), EDDFR35 (amino acids 1142–2799), EDDFR45 (amino acids 1976–2799) and EDDFR4 (amino acids 1976–2474) (Fig. 2A). Each of these fragments was cloned into the *pcDNA3.1*$^{+}$*-HisB* plasmid, which was further used to synthesize ^{35}S-radiolabeled EDD fragments using the rabbit reticulocyte lysate *in vitro* coupled transcription/translation system. The resulting ^{35}S-labeled proteins were assayed for their binding to a recombinant GST-Msp58 protein. Our results showed that EDDFR35, EDDFR45 and EDDFR4, which all contain the EDDFR4 region, were able to bind GST-Msp58, but not GST control (Fig. 2B). Therefore, we conclude that the region included in EDDFR4, located upstream of the HECT domain that contains the ubiquitin E3 ligase activity, is sufficient to interact with Msp58.

3.3. Msp58 directly interacts with EDD

The *in vitro* binding study shown in Fig. 2B used the EDD fragments synthesized with the rabbit reticulocyte lysate system. Given that other proteins present in the lysate may mediate the interaction between Msp58 and EDD, we sought to determine whether Msp58 directly interacts with EDD. To this end, we further characterized

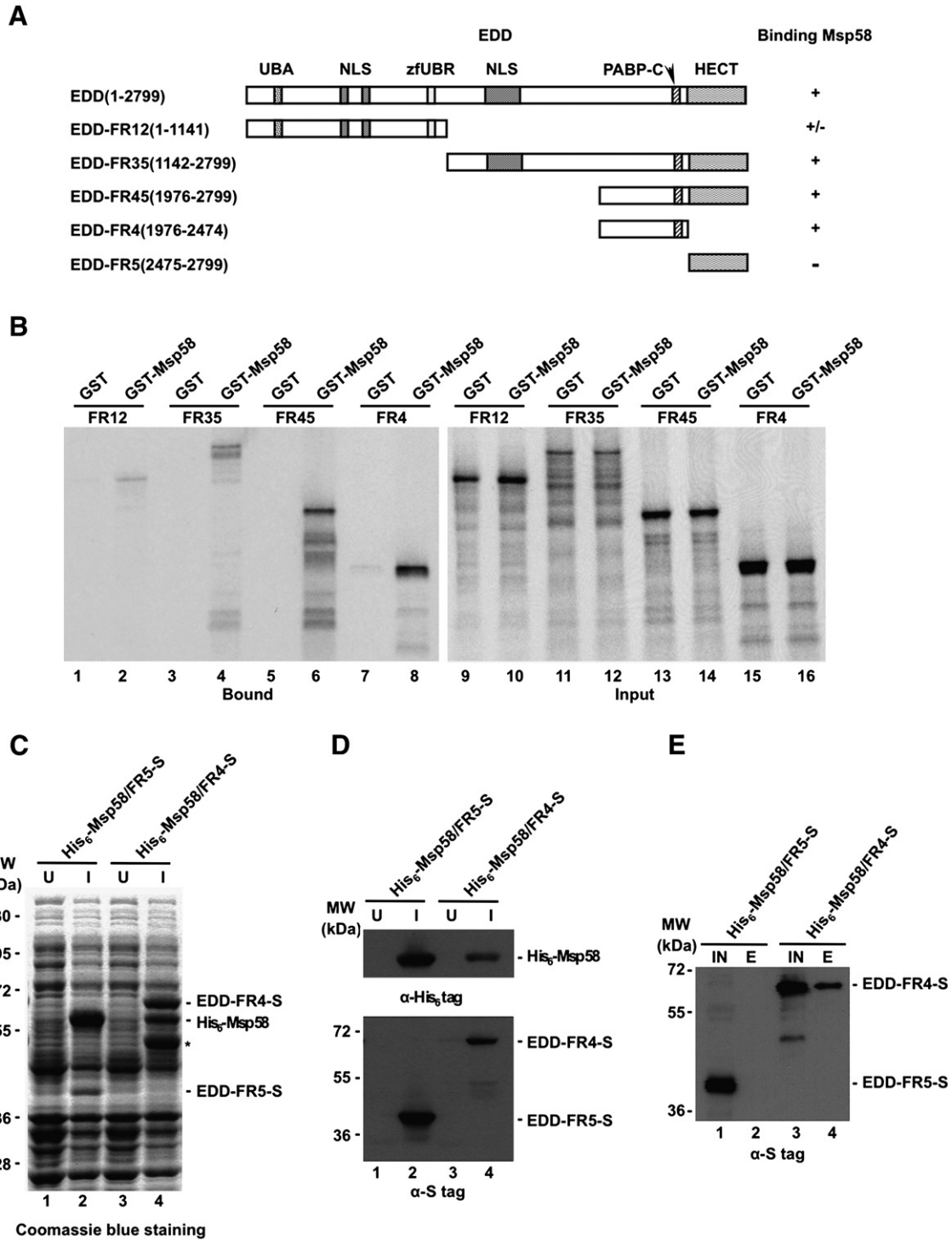

Fig. 2. Msp58 interacts with EDD via the EDDFR4 (1976–2474) region. (A) Schematic diagram of EDD and its derivative fragments that were used for *in vitro* binding assays. (B) The EDD fragments that contain the FR4 (1976–2474) region specifically interact with Msp58. Recombinant GST or GST-Msp58 proteins immobilized on the glutathione beads were incubated with ^{35}S-Met labeled EDD fragments (FR12, FR35, FR45 and FR4). The eluates were resolved by SDS-PAGE, followed by autoradiography. 15% of input was loaded to show the expression levels of proteins. (C) Coomassie blue staining of the lysates of uninduced (U, lanes 1 and 3) and induced (I, lanes 2 and 4) bacteria, co-expressing His_6-Msp58 with EDD-FR5-S or EDD-FR4-S. Asterisk points to a degradation product of EDD-FR4-S. (D) Immunoblotting of samples, as described in (C), with anti-His_6 tag or anti-S tag antibodies. (E) Msp58 directly binds EDD-FR4, but not EDD-FR5. Lysates of induced bacteria, as described in (C), were purified with cobalt beads. The input (IN, lanes 1 and 3) and the eluates (E, lanes 2 and 4) were immunoblotted with anti-S tag antibody.

their interaction using purified proteins. A bacteria-based co-expression system was used to simultaneously express recombinant Msp58 and EDDFR4. EDDFR5 was used as a negative control. In this experiment, the cDNAs coding for Msp58 and EDD fragments were cloned into *pETDuet-1* to express Msp58 and EDD fused with an amino-terminal His_6 tag and a carboxy-terminal S tag, respectively. The sequence-confirmed construct was transformed into *E. coli BL21(DE3)Codon Plus (RIL)* cells for optimal protein expression. The lysates of uninduced (lanes 1 and 3) and induced cells (lanes 2 and 4) were resolved by SDS-PAGE, followed by Coomassie blue staining (Fig. 2C) or

immunoblotting with anti-His tag and anti-S tag antibodies (Fig. 2D). The results showed that His_6-Msp58 and EDDFR4-S (or EDDFR5-S) were properly expressed as expected. The induced bacterial lysates were incubated with cobalt beads to purify His_6-tagged proteins. After extensive wash, the bound proteins were eluted with imidazole. As shown in Fig. 2E, EDDFR4-S (lane 4), but not EDDFR5-S (lane 2), was co-purified with His_6-Msp58. Thus, our bacteria-based binding assay proved that Msp58 directly interacted with EDD.

3.4. Two separate regions of Msp58 are sufficient to bind EDD

To characterize the interaction between Msp58 and EDD, we also mapped the domain of Msp58 that binds EDD (Fig. 3A). The cDNAs that encode different fragments of Msp58, amino acids 1–296 and 297–462, were cloned into a derivative of *pETDuet-1* vector to co-express His_6-tagged Msp58 fragments with EDDFR4-S. Upon the confirmation of their expression by immunoblotting (lanes 4 and 6, Fig. 3B), the bacterial lysates were incubated with S-protein agarose to pull down EDDFR4-S. Our results showed that full length Msp58 and $Msp58_{297–462}$ (lanes 2 and 6, Fig. 3C), but not $Msp58_{1-296}$ (lane 4, Fig. 3C), were co-purified with EDDFR4-S. These results demonstrated that the C-terminal region of Msp58 (amino acids 297–462) was sufficient to bind EDD.

$Msp58_{297–462}$ contains a coiled-coil motif (amino acids 301–350) and a forkhead-associated domain (FHA, amino acids 362–432) (Fig. 3A), both known to mediate protein–protein interactions. Since EDD has been shown to interact with the FHA domain of checkpoint kinase CHK2 [16], we tested the possibility that EDD also binds Msp58 via its FHA domain. We generated two additional constructs that co-express EDDFR4 with $Msp58_{1–342}$ or $Msp58_{343–462}$, the latter mainly consisting of the FHA domain (amino acids 362–432) [1]. Immunoblotting analyses confirmed that the recombinant proteins were appropriately expressed (lanes 2 and 4, Fig. 3D). Using the aforementioned approach, we showed that both $Msp58_{1–342}$ and $Msp58_{343–462}$ were able to bind EDDFR4 (lanes 4 and 6, Fig. 3E). $Msp58_{1-296}$ was used as a negative control (lane 2, Fig. 3E). These data indicate that the FHA domain and the region containing a coiled-coil motif were each sufficient to interact with EDD.

3.5. Msp58 co-localizes with EDD in the nucleoplasm

We showed that Msp58 and EDD were present in a protein complex purified from nuclear extracts (Fig. 1). In addition, results from studies using reticulocyte lysate-translated and bacterially expressed recombinant proteins demonstrated that Msp58 directly interacts with EDD. We thus wanted to know whether Msp58 co-localizes with EDD *in vivo*. We first analyzed their individual localizations by indirect immunofluorescence and confocal microscopy. HeLa cells were co-immunostained with mouse monoclonal mAb414 antibody, which recognizes several nuclear pore proteins at the nuclear envelope, and rabbit polyclonal antibodies against Msp58 (Fig. 4A, top) or EDD (Fig. 4A, bottom). Both Msp58 and EDD were predominantly detected in the nucleoplasm. Then, we addressed whether Msp58 and EDD colocalize in different mammalian cell types using two approaches. First, we examined the localizations of endogenous proteins in WI-38 (Fig. 4B, top) and HeLa (Fig. 4B, bottom) cells with rabbit and mouse polyclonal antibodies against Msp58 and EDD, respectively. Second, we transiently transfected HeLa cells with GFP-Msp58, followed by immunostaining with rabbit polyclonal antibodies against EDD. The results from both cell types consistently showed that (i) Msp58 was mainly located in the nucleus with limited amounts in the cytoplasm, (ii) EDD was predominantly localized in the nucleoplasm and nucleoli, and (iii) Msp58 co-localized with EDD in the nucleoplasm (Fig. 4).

3.6. EDD regulates the protein level and stability of Msp58

EDD is an E3 ubiquitin ligase and several substrates of EDD have been reported [18,24–27]. Since we showed that Msp58 directly interacted with EDD, we asked whether the protein level of Msp58 is also controlled by EDD. siRNAs targeting EDD were transfected into WI-38, HeLa and HEK293T cells, and GFP siRNAs were used as control. Seventy-two hours after transfection, the cells were harvested for immunoblotting analyses of Msp58 and EDD. Compared with control, depletion of EDD led to a significant accumulation of Msp58 in all analyzed cell lines (Fig. 5A). As these results indicated that EDD negatively regulates Msp58, we next examined whether EDD affects Msp58 protein turnover. HeLa cells transfected with either EDD or GFP siRNAs were harvested at different time points (0–24 h) after protein synthesis was blocked by cycloheximide (CHX) and analyzed by immunoblotting (Fig. 5B). Quantification of immunoblots indicates that knockdown EDD significantly increased Msp58's half-life from ~8 to ~20 h (Fig. 5C). Taken together, these results strongly support our view of EDD as a negative regulator of Msp58.

3.7. Msp58 is degraded by the ubiquitin–proteasome pathway

The ubiquitin–proteasome pathway plays a crucial role in protein degradation. As our previous data showed that Msp58 turnover is negatively controlled by E3 ubiquitin protein ligase EDD (Fig. 5), we therefore further investigated whether Msp58 is regulated by the ubiquitin–proteasome pathway. To determine whether the proteasome is involved in the degradation of Msp58, we treated WI-38, HeLa and HEK293T cells with proteasome inhibitor MG132 and examined its effect on the protein levels of Msp58, by immunoblotting. In these experiments, cells were also treated with cycloheximide to inhibit nascent protein synthesis. As expected, Msp58 protein levels in cycloheximide treated cells were significantly reduced (Fig. 6A, lanes 2, 5 and 8), but the levels of Msp58 were partially restored in cells simultaneously treated with cycloheximide and MG132 (Fig. 6A, lanes 3, 6 and 9). These results demonstrate that Msp58 undergoes proteasome-mediated degradation.

As protein degradation by the proteasome requires the substrates to be post-translationally modified by ubiquitination, we next studied whether Msp58 is ubiquitinated and the role of EDD E3 ubiquitin ligase in this process. We used the *in vivo* ubiquitination assay, where lysates from HEK293T cells co-transfected with HA-ubiquitin and GFP-Msp58 (or control GFP) expression constructs, with or without EDD siRNAs, were immunoprecipitated with anti-HA affinity matrix (Fig. 6B). The eluates (lanes 4–6 and 10–12), as well as the inputs (lanes 1–3 and 7–9), were then examined by immunoblotting with antibodies against Msp58 (lanes 1–6) and GFP (lanes 7–12). Our results show that the exogenously expressed GFP-Msp58 was ubiquitinated, but the level of ubiquitinated GFP-Msp58 was not significantly affected by silencing EDD.

3.8. Msp58 and EDD regulate cyclin levels and cell cycle progression

In the knockdown studies, we noticed that depletion of Msp58 resulted in a significant increase in the number of viable cells, whereas silencing EDD displayed an opposite phenotype. We reasoned that this might be, at least in part, due to the changes of Msp58 protein level, since Msp58 has been shown to affect cell cycle progression through its known effects on cyclin gene expression [8,9]. To explore this hypothesis, we used propidium iodide labeling and flow cytometry to examine cell cycle phases in WI-38 cell cultures treated with siRNAs to EDD, to Msp58, or to both. Seventy-two hours after transfection with siRNAs, subconfluent cells were harvested and analyzed (Fig. 7, Table 1). The results showed that, compared with the control (GFP siRNA-transfected cells), depletion of EDD did not significantly affect G1, but led to a 66% rise in the number of cells in S and a 38% decrease in the G_2/M population. Silencing Msp58 reduced the percentage of

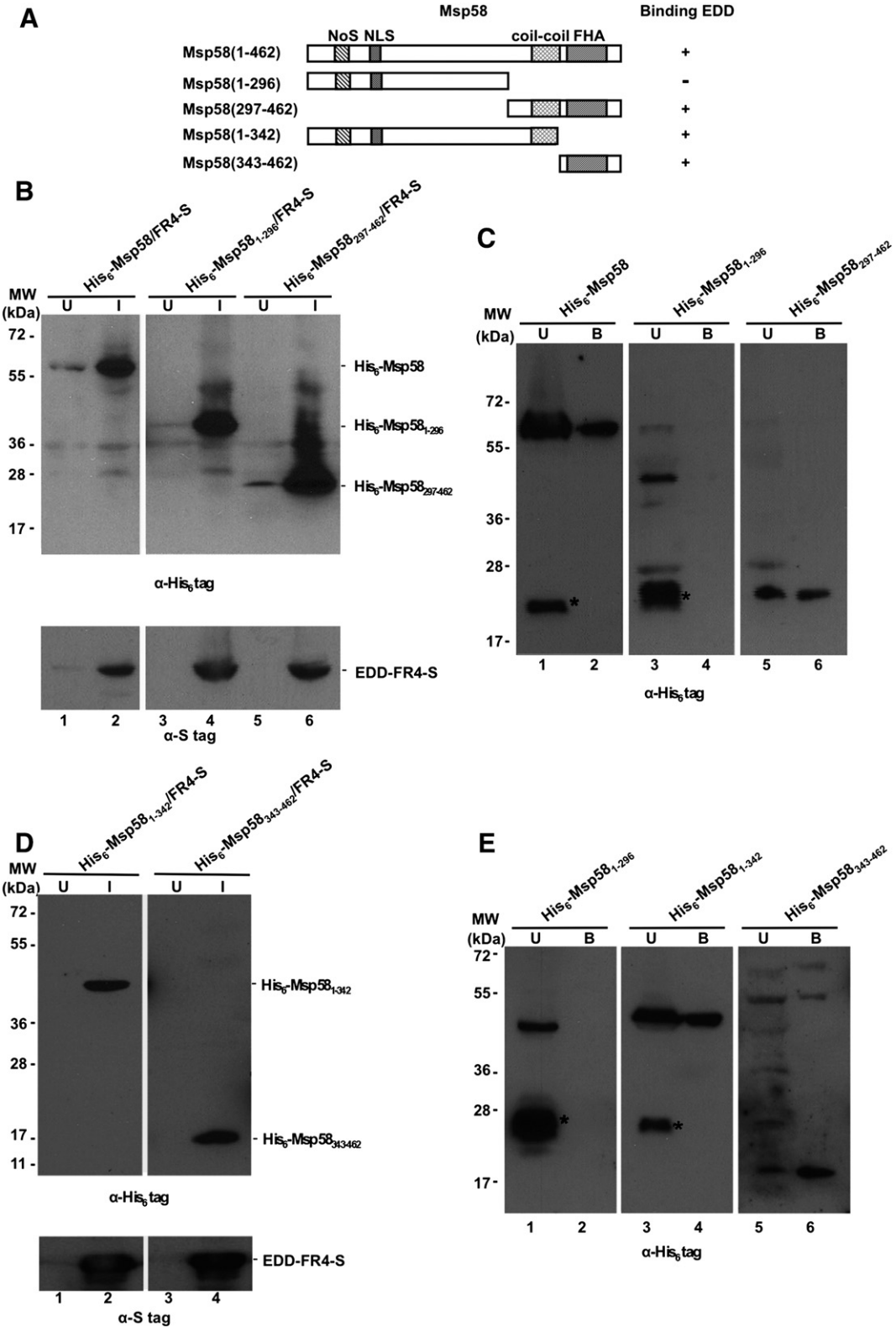

Fig. 3. Two independent regions of Msp58 can bind EDD. (A) Schematic diagram of Msp58 with predicted domains and its derivative fragments used to test their binding with EDD. (B) The lysates of uninduced (U, lanes 1, 3 and 5) and induced (I, lanes 2,4 and 6) bacteria, co-expressing EDDFR4-S with His_6-Msp58 (or His_6- $Msp58_{1-296}$ or His_6- $Msp58_{297-462}$), were resolved by SDS-PAGE, followed by immunoblotting with anti-His_6 tag and anti-S tag antibodies. (C) $Msp58_{297-462}$ contains an EDD interacting domain. S-protein agarose was incubated with the lysate from bacteria co-expressing EDDFR4-S with His_6-Msp58 (or Msp58 fragments). The unbound (U, lanes 1, 3 and 5) and bound (B, lanes 2, 4 and 6) proteins were examined by immunoblotting with anti-His_6 tag antibody. Asterisks point to degradation fragments. (D) The lysates of uninduced (U, lanes 1 and 3) and induced (I, lanes 2 and 4) bacteria, co-expressing EDDFR4-S with His_6-$Msp58_{1-342}$ (lanes 1 and 2) or $Msp58_{343-462}$ (lanes 3 and 4), were examined as described in (B). (E) Both His_6-$Msp58_{1-342}$ (lane 4) and $Msp58_{343-462}$ (lane 6) are able to bind EDD. The experiments were performed as described in (C). His_6-$Msp58_{1-296}$ (lanes 1 and 2) served as a negative control. Asterisks point to degradation fragments.

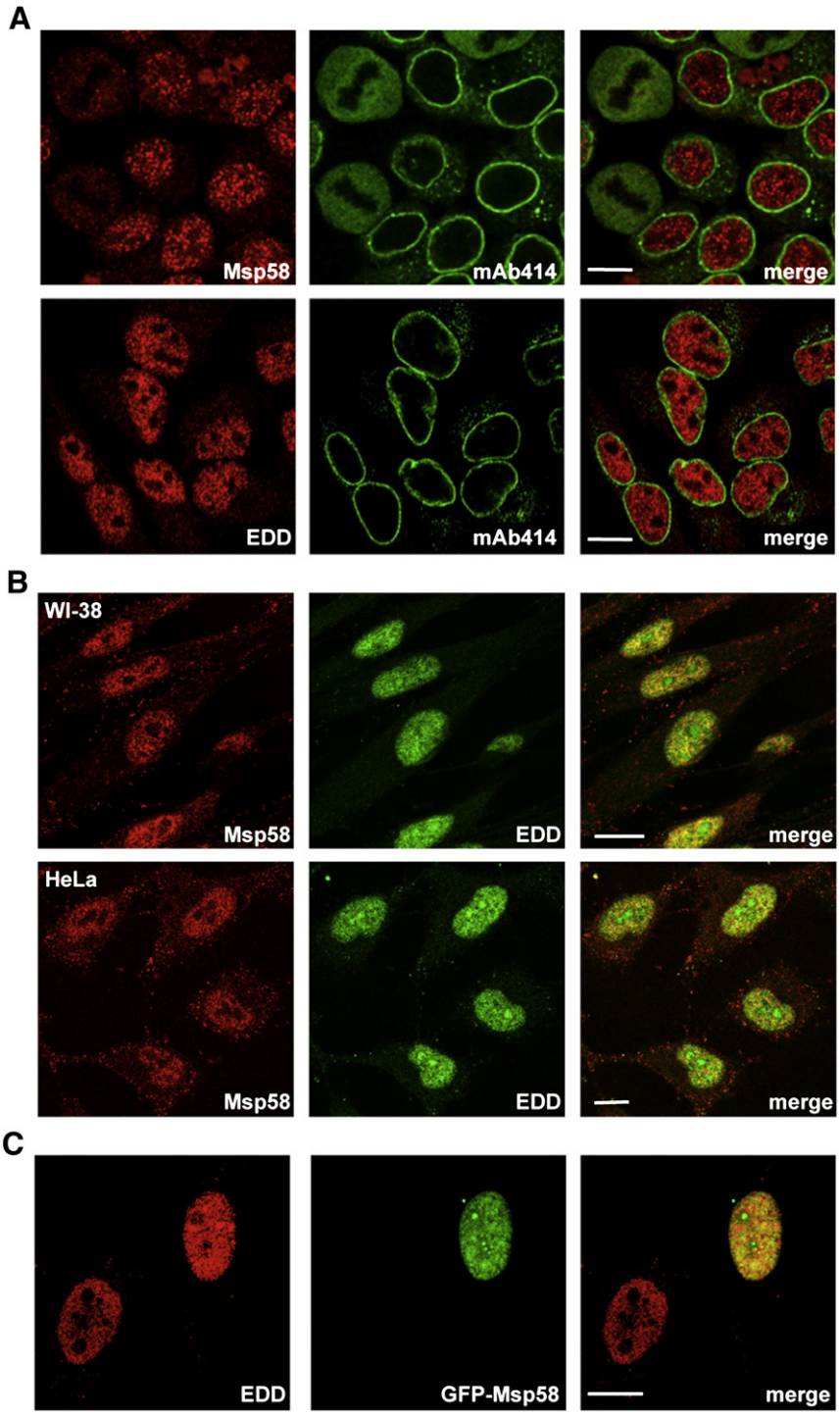

Fig. 4. EDD co-localizes with Msp58 in the nucleoplasm of mammalian cells. (A) Immnunostaining of HeLa cells with mouse monoclonal antibody mAb414 and rabbit polyclonal anti-Msp58 (top) or anti-EDD (bottom) antibodies. (B) WI-38 (top) and HeLa (bottom) cells were immunostained with rabbit polyclonal anti-Msp58 and mouse polyclonal anti-EDD antibodies. (C) HeLa cells transfected with GFP-Msp58 expression construct were immunostained with the rabbit polyclonal antibodies against EDD. Bars: 10 μm.

cells in G1 (down 24%). As in the EDD knockdown, there was an increase in the percentage of cells in S (up 55%), but most notably, there was an increase (not a decrease) in the G_2/M population (up 50%) (Table 1, Fig. 7B). Co-depletion of Msp58 and EDD brought both G1 and G2/M populations back to control levels, but retained a small increase in the S population (up 25%) (Table 1). Overall, our data indicated that individual depletion of Msp58 or EDD affected different phases of the cell cycle, while simultaneous depletion partially reverted these phenotypes.

Msp58 has been implicated in regulating the expression of several cyclins [8,9,28]. As significant changes in cell cycle progression were observed in Msp58- or EDD-depleted cells, we therefore examined whether the levels of cyclins were affected. As shown in Fig. 7C, EDD depleted cells showed an increase of cyclins D1 and E, whereas knocking down Msp58 resulted in an accumulation of cyclins B and D3. When both Msp58 and EDD were depleted, the levels of cyclins B, D1 and E reverted to levels similar to those in control samples.

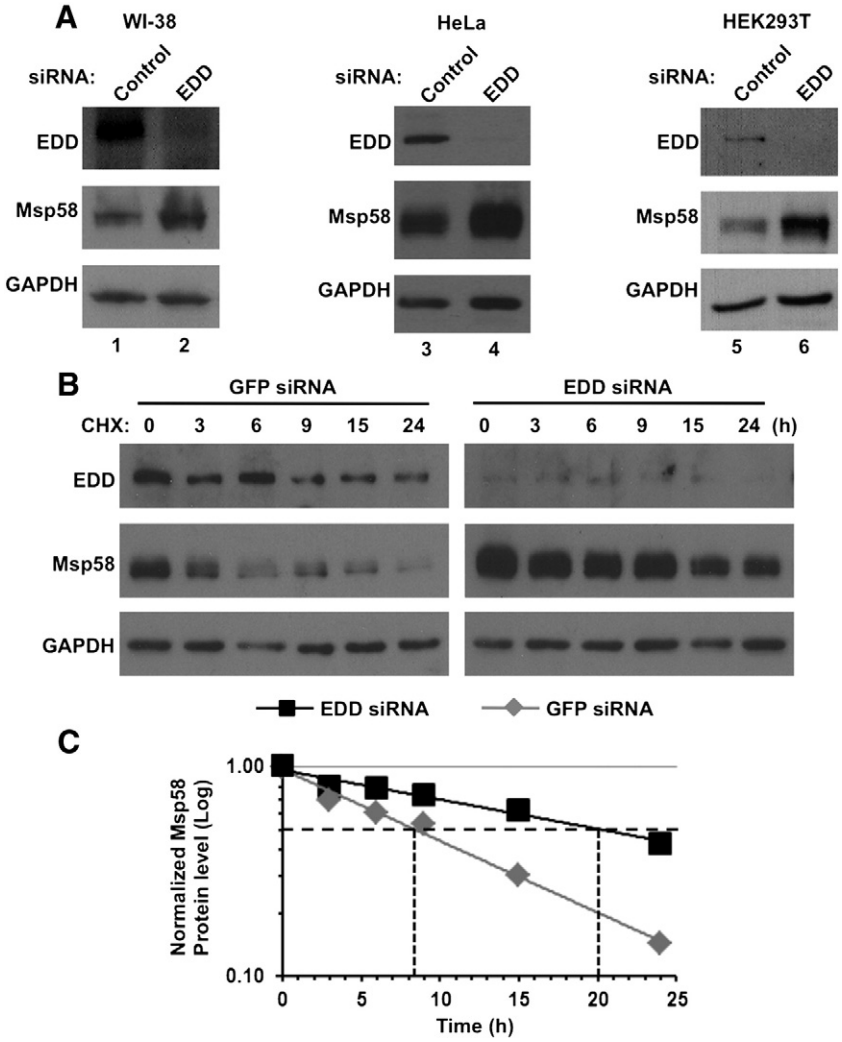

Fig. 5. EDD negatively regulates the protein level and stability of Msp58. (A) Depletion of EDD resulted in higher Msp58 protein levels in WI-38, HeLa and HEK293T cells. Seventy-two hours after transfection with either EDD (lanes 2, 4 and 6) or control (lanes 1, 3 and 5) siRNAs, the cells were harvested and analyzed by immunoblotting with the specified antibodies. (B) EDD knockdown increased Msp58 protein stability. HeLa cells were transfected with EDD or control siRNAs, and 72 h after transfection cells were treated with CHX. At the indicated time points, cells were harvested and analyzed by immunoblotting. (C). A semi-log graph showing the half-lives of Msp58 as shown in (B). The trendlines are derived from the raw data regression.

These results suggest that Msp58 and EDD play a role in modulating cyclin expression and cell cycle progression.

4. Discussion

Msp58's roles in transcriptional regulation and cell proliferation remain poorly understood. In this work, we have identified ubiquitin E3 ligase EDD as a novel interacting partner of Msp58, and shown that Msp58 and EDD colocalize in the nucleus of mammalian cells. Since we found that the level of Msp58 was increased in EDD depleted cells, one possibility is that EDD ubiquitinates Msp58, leading to its degradation through the ubiquitin–proteasome pathway. Consistent with this hypothesis, we showed an accumulation of Msp58 in cells treated with the proteasome inhibitor MG132 (Fig. 6A). Others have seen similar effects on Msp58 in cells with impaired proteasomal activity [4]. In addition, we demonstrated the ubiquitination of ectopically expressed GFP-Msp58 in HEK293T cells, which, interestingly, was not affected by knocking down EDD (Fig. 6B). This shows that ubiquitination of exogenous Msp58 can occur in an EDD-independent manner. However, as low protein levels precluded the study of endogenous Msp58 under our experimental conditions, it remains possible that EDD's effect on Msp58 protein levels is through direct ubiquitination. Notably, EDD (also called UBR5) is a member of a family of E3 ubiquitin ligases known as N-recognins, which target substrates that contain N-degrons for their degradation via the N-end rule pathway. EDD recognizes type 1 N-degron (11). A putative degron sequence at the N-terminus of Msp58 was disrupted by the insertion of GFP, perhaps explaining the absence of EDD-mediated ubiquitination of this ectopically expressed protein. Currently, we are investigating the mechanism by which EDD controls the protein level of endogenous Msp58.

An alternative but not mutually exclusive possibility is that Msp58 serves as an adaptor protein for recruiting other substrates to EDD. Indeed, previous work provides an example of this idea: the adaptor protein VPRBP recruits the substrate katanin p60 to the EDD ubiquitin ligase complex [26]. During mapping of the EDD-interacting region of Msp58, we found to our surprise that two domains of Msp58 ($Msp58_{1–342}$ and $Msp58_{343–462}$) were each sufficient to bind EDD (Fig. 3). $Msp58_{1–342}$ contains a coiled-coil motif and $Msp58_{343–462}$ has an FHA (forkhead-associated) domain; both are known to mediate protein–protein interactions. We speculate that, with two EDD-binding regions, Msp58 has more flexibility to interact with different proteins while still maintaining its ability to bind EDD. With this capability, Msp58 would be able to recruit a broad range of proteins to EDD for ubiquitination, and thus regulate their stability, localization and/or

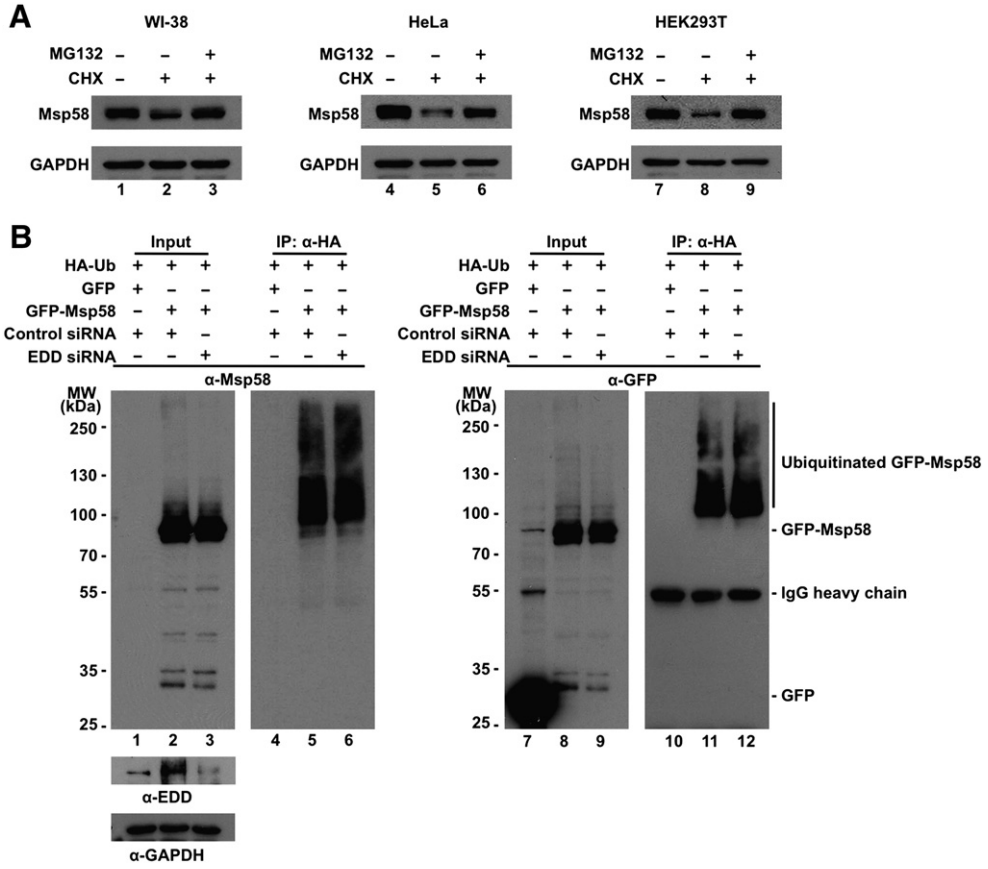

Fig. 6. Msp58 is regulated by the ubiquitin–proteasome pathway. (A) Msp58 protein level is controlled by the proteasome. Before harvest, WI-38, HeLa and HEK293T cells were treated with cycloheximide (CHX; lanes 2, 3, 5, 6, 8 and 9), with (lanes 3, 6 and 9) or without (lanes 2, 5 and 8) MG132, for 6 h. The lysates were analyzed by immunoblotting with specified antibodies. (B) GFP-Msp58 was ubiquitinated *in vivo* and knockdown of EDD had no obvious effect on the level of ubiquitinated GFP-Msp58. HEK293T cells were co-transfected with HA-ubiquitin and GFP-Msp58 expression constructs, with or without EDD siRNAs. Cells co-transfected with HA-Ubiquitin and GFP were used as control. Lysates were immunoprecipitated with anti-HA affinity matrix and the inputs (lanes 1–3 and 7–9) and eluates (lanes 4–6 and 10–12) were examined by immunoblotting with specified antibodies.

functions. As described in the Introduction, Msp58 was identified as an interacting partner of several proteins involved in diverse nuclear processes [1–6]. We are currently examining whether some of these Msp58-associated proteins undergo EDD-mediated ubiquitination.

As previously mentioned, two independent regions of Msp58 ($Msp58_{1–342}$ and $Msp58_{343–462}$) are able to interact with EDDFR4 fragment (amino acids 1976–2474). EDDFR4, located upstream of the HECT domain that contains the ubiquitin E3 ligase activity, contains a carboxyl region of poly (A)-binding protein (PABP)-like domain (PABP-C, amino acids 2391–2455) and overlaps with the region (amino acids 889–2526) that interacts with the FHA domain of checkpoint kinase CHK2. Msp58 also contains an FHA domain (amino acids 362–432) and our studies demonstrated that $Msp58_{343–462}$ is sufficient to bind EDDFR4. Therefore, it is possible that Msp58 competes with CHK2 for the same binding region of EDD. Future studies will be carried out to test this hypothesis.

Our results showed that both increases and decreases in Msp58 protein affected the expression of several cyclins and cell cycle progression. Msp58 depleted cells showed an accumulation of cyclins B and D3 and a decrease of cyclin E (Fig. 7C). At the same time, these cells showed a decrease in G1 and an increase in S and G2/M populations (Table 1, Fig. 7B). D3 is a G1 cyclin that promotes the transition from G1 to S, while cyclin B is up in G2 and M phases and must be degraded for cells to exit M and enter the G1 phase. The up-regulation of cyclins D3 and B in Msp58 depleted cells, therefore, is consistent with their cell cycle profile (an increase in both S and G2/M and a decrease in G1). Recently, Hsu et al. reported that overexpression of Msp58 in human fibrosarcoma HT1080 cells down-regulates the levels of cyclins A, B1, D and E2 and Msp58 controls cell proliferation in a cell-type dependent manner[9].

Depleting EDD led to an increase of Msp58, low cyclin B and a reduced G2/M compartment (Fig. 7, Table 1). This is the opposite phenotype to that of Msp58 depletion, suggesting an inverse relationship between Msp58 levels and accumulation of cells at the G2/M phase of the cell cycle. Certainly, we cannot assume that all the cell cycle effects seen upon EDD depletion are due only to the increase in Msp58 levels, since EDD previously has been shown to regulate cell cycle progression [12–14]. Nevertheless, it is clear that a decrease in Msp58 modifies cell cycle progression, and since EDD determines whether Msp58 protein levels are high or low, the Msp58/EDD interaction must serve an important regulatory role in this process.

The changes in cyclin levels that accompanied Msp58 depletion (and Msp58 increases caused by EDD depletion) are consistent with earlier studies that showed that MCRS2, a *Drosophila* homolog of human Msp58, stimulates transcription of a subset of cyclin genes by recruiting the RNA polymerase II complex to their promoters [8]. Msp58 is likely to affect the cell cycle through other mechanisms as well. For example, Meunier and Vernos have recently reported that Msp58/MCRS1 is required for spindle assembly and regulates the stability of kinetochore spindles [7]. Aberrant expression of Msp58 results in cell cycle arrest in mitosis [7].

Surprisingly, when EDD was co-silenced with Msp58, the depletion of Msp58 was less pronounced. We interpret this as further evidence of EDD's negative effect on Msp58; both EDD and Msp58 siRNA contribute to the depletion of Msp58 protein in Msp58 knockdown samples. In co-depleted cells, EDD's regulatory function was compromised, allowing

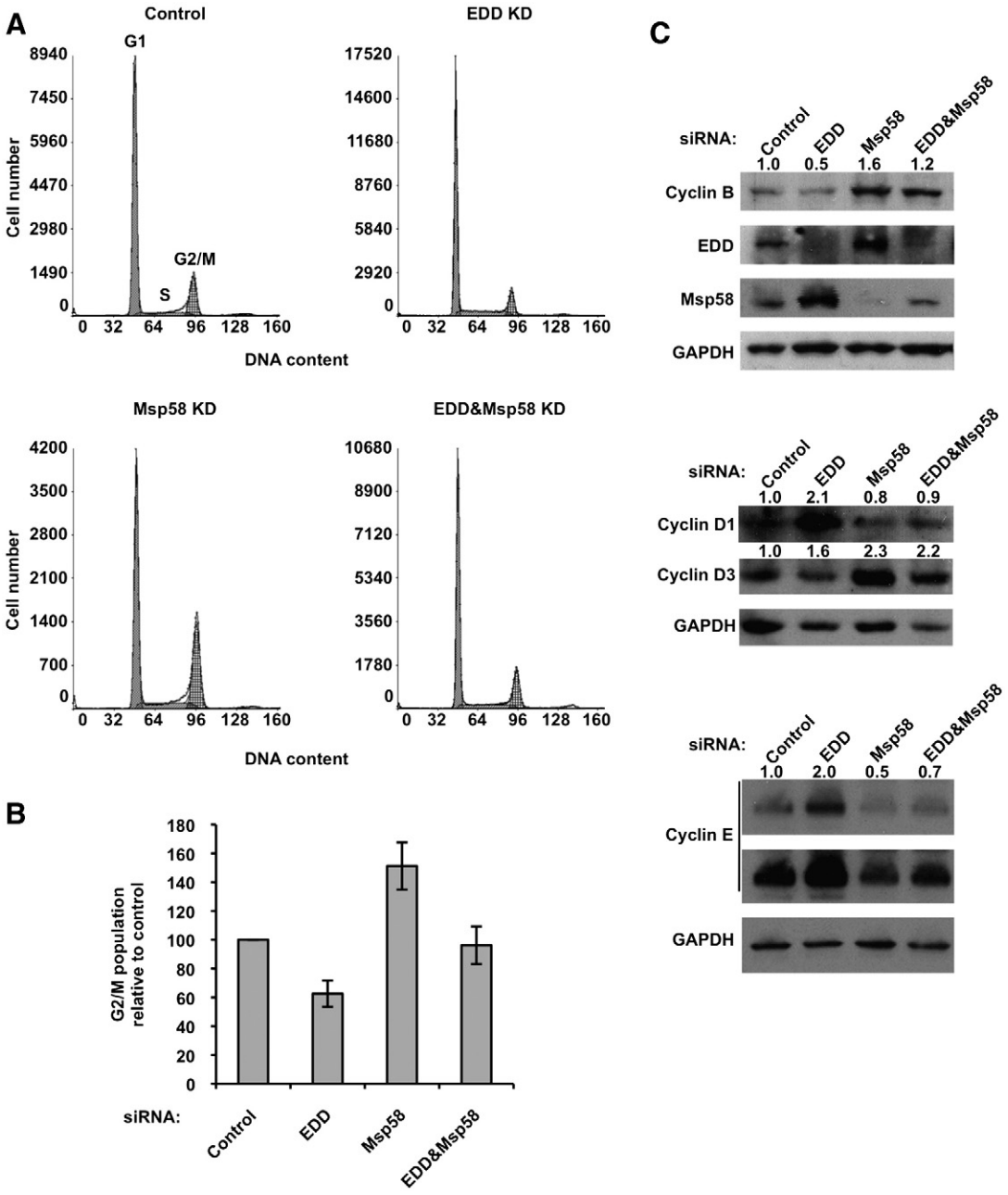

Fig. 7. Msp58 and EDD regulate cell cycle progression. (A) Representative FACS histograms of WI-38 cells transfected with specified siRNAs. (B) Analysis of the G2/M phase of the cell cycle. Bars show relative populations of WI-38 cells transfected with specified siRNAs as compared with the cells transfected with GFP siRNAs. Data presented as means ± SD ($n = 5$). (C) Representative immunoblots showing the levels of cyclins (B, D1, D3, and E) in different siRNA-transfected samples. The protein bands were semi-quantified using ImageJ software, and the numbers on top of blots represent the relative levels of cyclins normalized to control siRNA-transfected sample.

accumulation of residual Msp58. Consistent with the increase of Msp58 protein to near-control levels in the co-depletion sample, the G2/M population also largely returned to normal. These findings further confirm EDD's ability to negatively regulate Msp58 and the importance of this Msp58/EDD interaction to cell cycle progression.

Table 1
Msp58 and EDD regulate cell cycle progression.

siRNAs	Phases	G1	S	G2–M
Control		68.5 ± 3.7	10.7 ± 1.3	20.7 ± 3.3
EDD		69.4 ± 4.0	17.8 ± 3.2*	12.9 ± 2.1*
Msp58		52.1 ± 2.7*	16.6 ± 4.7*	31.3 ± 5.3*
EDD and Msp58		67.0 ± 2.6	13.4 ± 2.7*	19.6 ± 1.2

Note: $n = 5$; means ± SD. (*, $p < 0.05$ in comparison with control siRNA).

5. Conclusion

In this study, we have identified a physical and functional interaction between EDD and Msp58. EDD directly binds and regulates Msp58, and both proteins modulate cyclin levels and cell cycle progression. Since Msp58 and EDD are often aberrantly expressed in cancers [11,22,28,29], our findings shed light on the mechanism of their roles in tumorigenesis.

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